ISG15 as a novel tumor biomarker for drug sensitivity

Shyamal D. Desai,[1] Laurence M. Wood,[1] Yu-Chen Tsai,[1] Tao-Shih Hsieh,[2] Jeffrey R. Marks,[3] Georgia L. Scott,[3] Beppino C. Giovanella,[4] and Leroy F. Liu[1]

[1]Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey; Departments of [2]Biochemistry and [3]Surgery, Duke University Medical Center, Durham, North Carolina; and [4]Stehlin Foundation for Cancer Research, Houston, Texas

Abstract

Tumor cells are known to exhibit highly varied sensitivity to camptothecins (CPT; e.g., irinotecan and topotecan). However, the factors that determine CPT sensitivity/resistance are largely unknown. Recent studies have shown that the ubiquitin-like protein, IFN-stimulated gene 15 (ISG15), which is highly elevated in many human cancers and tumor cell lines, antagonizes the ubiquitin/proteasome pathway. In the present study, we show that ISG15 is a determinant for CPT sensitivity/resistance possibly through its effect on proteasome-mediated repair of topoisomerase I (TOP1)-DNA covalent complexes. First, short hairpin RNA-mediated knockdown of either ISG15 or UbcH8 (major E2 for ISG15) in breast cancer ZR-75-1 cells decreased CPT sensitivity, suggesting that ISG15 overexpression in tumors could be a factor affecting intrinsic CPT sensitivity in tumor cells. Second, the level of ISG15 was found to be significantly reduced in several tumor cells selected for resistance to CPT, suggesting that altered ISG15 regulation could be a significant determinant for acquired CPT resistance. Parallel to reduced CPT sensitivity, short hairpin RNA-mediated knockdown of either ISG15 or UbcH8 in ZR-75-1 cells resulted in increased proteasomal degradation of CPT-induced TOP1-DNA covalent complexes. Taken together, these results suggest that ISG15, which interferes with proteasome-mediated repair of TOP1-DNA covalent complexes, is a potential tumor biomarker for CPT sensitivity. [Mol Cancer Ther 2008;7(6):1430–9]

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Note: Current address for S.D. Desai: Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112.

Requests for reprints: Leroy F. Liu, Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Phone: 732-235-4592; Fax: 1-732-235-4073. E-mail: lliu@umdnj.edu; Shyamal D. Desai, Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112. E-mail: sdesai@lsuhsc.edu

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Introduction

Two camptothecin (CPT) analogues, irinotecan (Camptosar) and topotecan (Hycamtin), have been developed and used in the clinic for the treatment of certain human cancers (e.g., ovarian and colorectal cancers; refs. 1–3). However, it has been shown that not all cancers are sensitive to CPT-based chemotherapy (4, 5). It is of particular significance that *in vitro* CPT sensitivity also varies greatly in a panel of breast and colon cancer cell lines (6–8). To date, the factors affecting tumor sensitivity to CPT are not well understood.

CPT kill tumor cells by trapping topoisomerase I (TOP1)-DNA covalent complexes (9, 10). The repair of this unique type of DNA damage, which is not fully understood, is expected to affect CPT sensitivity/resistance (7, 11, 12). Recent studies have shown that an ubiquitin/26S proteasome pathway is activated by CPT and this leads to the degradation of TOP1-DNA covalent complexes in CPT-treated cells (13–15). It has been suggested that proteasomal degradation of TOP1 (TOP1 down-regulation) represents a repair mechanism for CPT-induced TOP1-DNA covalent complexes (13, 14). Indeed, cells proficient in this repair process are more resistant to CPT (7). In addition, overexpression of Cullin3, a component of a SCF complex, has been shown to increase ubiquitination and TOP1 down-regulation, resulting in CPT resistance (16). Furthermore, cotreatment with the proteasome inhibitor, MG132, which inhibits TOP1 down-regulation, increases tumor cell sensitivity to CPT (7). These results suggest that the ubiquitin/proteasome pathway could be an important determinant for CPT sensitivity/resistance.

Several studies have shown that CPT-induced TOP1 down-regulation is defective in many tumors, a factor that could contribute to tumor-specific killing by CPT. In tissue culture models, CPT has been shown to induce TOP1 down-regulation in normal nontransformed cells but not in many tumor cells (7). Similarly, in a nude mouse model, topotecan treatment has been shown to cause TOP1 down-regulation in many normal tissues (e.g., blood, brain, kidney, liver, and skin) but not in xenografted MDA-MB-435 breast cancer cells (14). Furthermore, patients receiving topotecan therapy also exhibit reduced TOP1 levels in normal peripheral blood cells but not in leukemic cells (17, 18). Thus, TOP1 down-regulation in normal tissues has been suggested to be a cellular response to evade the toxic effect of TOP1-directed anticancer drugs (7). Most tumor cells are defective in CPT-induced TOP1 down-regulation, which could explain in part the increased sensitivity of tumor cells to CPT (7, 19).

The molecular basis for the defective proteasomal degradation of TOP1 in many tumor cells is not clear.

However, recent studies have shown that the ubiquitin-like protein, IFN-stimulated gene 15 (ISG15), is highly elevated, but variably expressed, in many tumors (20–22). In addition, high-level expression of ISG15 has been shown to interfere with the ubiquitin/26S proteasome pathway, leading to altered degradation of many cellular proteins (20). ISG15 is composed of two ubiquitin homology domains connected by a small linker region (23, 24). Each homology domain is ~30% homologous to ubiquitin (25–27). The carboxyl terminus of ISG15 retains the canonical LRLRGG ubiquitin sequence required for its conjugation to intracellular targets (28–30). ISG15 is conjugated to its substrates in much the same way as ubiquitin, requiring E1, E2, and E3, all of which are induced by type I IFN (23, 27). The E1 for ISG15, UBE1L, is specific for ISG15 (31). However, the E2 (that is UbcH8; refs. 32, 33) and E3s (that is, Rsp5, Herc5, and Efp; refs. 32, 34–36) for ISG15 are dual-functional E2/E3 needed for both protein ISGylation and ubiquitination. It has been suggested that ISGylation, which is elevated in many tumors, interferes with ubiquitination through substrate competition at the E2/E3 level (20).

In the present study, we test the hypothesis that the ISG15 conjugation pathway is a determinant for tumor cell sensitivity to CPT by antagonizing CPT-induced TOP1 down-regulation. By knocking down either ISG15 or UbcH8 (the major E2 for ISG15; ref. 32), the ISG15 pathway in breast cancer ZR-75-1 cells was shown to be an important determinant for CPT sensitivity by interfering with TOP1 down-regulation. In addition, ISG15 expression was also shown to be significantly reduced in several tumor cells selected for CPT resistance. These results suggest that ISG15 and factors in its conjugation pathway could serve as tumor biomarkers for CPT sensitivity/resistance.

Materials and Methods

Cells

All cells were cultured in RPMI supplemented with 10% Fetalplex (Gemini Bio-Products), L-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 37°C incubator with 5% CO_2. Breast cancer ZR-75-1 cells stably expressing ISG15 or UbcH8 short hairpin RNA (shRNA) were maintained in hygromycin B (100 μg/mL).

Immunoblotting

Cells (5×10^5) were plated in 35 × 10 mm tissue culture dishes and incubated at 37°C in a CO_2 incubator. One day after plating, cells were lysed with 2× SDS gel sample buffer. After boiling for 10 min, cell lysates were sonicated and subjected to analysis by SDS-PAGE (6% and 15% for determination of TOP1 and ISG15, respectively). Proteins were transferred onto nitrocellulose membrane. Immunoblotting was carried out using various antibodies followed by the enhanced chemiluminescence Western procedure (Pierce). The signals were detected and quantified by the Kodak Image Station 2000R. Equal protein loading was assessed by reprobing the same membrane with anti-tubulin antibody.

Band Depletion Assay for TOP1 Cleavage Complexes

Cells (10^6 per sample) were treated with CPT (25 μmol/L in 1% DMSO) for various times at 37°C. Cells were then lysed with 0.2 N NaOH containing 2 mmol/L EDTA (14). Cell lysates were then neutralized with 1:10 volume of a solution containing 10% NP-40, 1 mol/L Tris (pH 7.4), 0.1 mol/L $MgCl_2$, 0.1 mol/L $CaCl_2$, 10 mmol/L DTT, 1 mmol/L EGTA, and a mixture of peptide protease inhibitors (100 μg/mL each of leupeptin, pepstatin, and aprotinin) followed by the addition of another 1:10 volume of 2 N HCl. Reactions were terminated by the addition of SDS-PAGE sample buffer. Immunoblotting analysis of cell lysates was carried out using anti-Scl-70 antibody from scleroderma patients as described above.

CPT-Induced TOP1 Down-Regulation

TOP1 down-regulation was measured as described above with two modifications. First, cells were incubated in CPT-free medium for 30 min before lysis (to reverse TOP1 cleavage complexes and to deconjugate TOP1-ubiquitin and TOP1-UBL conjugates). Second, neutralized cell lysates were incubated with *Staphylococcus aureus* nuclease S7 (60 units/reaction) for 20 min on ice (to release TOP1 from residual TOP1-DNA covalent complexes) before termination with SDS-PAGE sample buffer.

Construction of ISG15 and UbcH8 shRNA Vectors

ISG15 shRNA Vector. The pSilencer 4.1 CMV-hygro shRNA expression vector (Ambion) was used for constructing ISG15-shRNA expression vector. Sense (5′-GATCCTGCGACGAACCTCTGAACATTCAAGAGATTACGCTGCTTGGAGACTTGTA-3′) and antisense (5′-AGCTTACAAGTCTCCAAGCAGCGTAATCTCTTGAATGTTCAGAGGTTCGTCGCAG-3′) shRNA oligonucleotides, targeting the 232 to 250 region of ISG15 (accession no. AY168648), were synthesized (IDTDNA). The oligonucleotides were diluted in TE (10 mmol/L Tris, 1 mmol/L EDTA) to ~1 μg/mL. The sense and antisense oligonucleotides were annealed by heating to 90°C for 30 min in an annealing solution (provided by the manufacturer) followed by incubation at 37°C for 1 h. The annealed DNA was ligated into the pSilencer 4.1 CMV-hygro vector DNA cut with *Bam*HI and *Hin*dIII restriction enzymes followed by transformation into *Escherichia coli* DH5α. Plasmid DNA was purified and sequenced using the supplied primer sequences (5′-AGGCGATTAAGTTGGGTA-3′ and 5′-CGGTAGGCGTGTACGGTG-3′) for verification.

Ubch8 shRNA Vector. The pSilencer 4.1 CMV-hygro shRNA expression vector (Ambion) was used for constructing the UbcH8-shRNA expression vector. Sense (5′-GATCCCGAGAACGGACAGATTTGCTTCAAGAGATTGCTCTTGCCTGTCTAAACGA-3′) and antisense (5′-AGCTTCGTTTAGACAGGCAAGAGCAATCTCTTGAAGCAAATCTGTCCGTTCTCGG-3′) shRNA oligonucleotides, targeting the 237 to 255 region of UbcH8 (accession no. AF031141), were synthesized (IDTDNA). Construction and verification of UbcH8-shRNA vector were done as described above.

Control shRNA Vector. As a negative control, the pSilencer 4.1 CMV-hygro negative control plasmid was

used (Ambion). The plasmid encodes a shRNA whose sequence is not found in the mouse, human, or rat genome databases.

Construction of shRNA Stable Transfectants

Breast cancer ZR-75-1 cells (1×10^6) were transfected with 4 μg each of XMNI-linearized ISG15, UbcH8, or control shRNA vectors using the Polyfect transfection reagent (Qiagen). Following incubation for 72 h, cells were washed with fresh RPMI and incubated at 37°C for 24 h. Cells were trypsinized and replated into 150 mm tissue culture plates. Selection medium that contained 200 μg/mL hygromycin B was added to the cells 24 h after replating. Individual colonies were picked following 3 weeks of hygromycin selection and screened for ISG15 and UbcH8 expression by Western blotting analysis using anti-ISG15 and anti-UbcH8 antisera, respectively.

Cell Survival Assays

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. Cells (3,000 per well) were plated in 96-well microtiter plates and incubated at 37°C in a CO_2 incubator. For determination of IC_{50}, cells were treated with varying concentrations of CPT for 4 days. Cells were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C. The dye produced by viable cells was dissolved in DMSO and the absorbance measured at 570 nm. All assays were done at least twice in six replicate wells.

Clonogenic Assay. Cells (100-500 per plate) were plated in six-well tissue culture plates and incubated at 37°C in a CO_2 incubator. Cells were treated with various concentrations of CPT for 1 h. Following CPT treatment, cells were washed free of CPT and placed in fresh medium. After 10 days, survived colonies were stained with methylene blue and counted using the Minicount machine.

Cell Counting Assay. Logarithmically growing cells were treated with various concentrations of CPT for 1 h. CPT was removed by washing the plates with CPT-free medium four times. Washed cells were allowed to grow in CPT-free medium for 4 days. Cell survival was determined by counting trypsinized cells with the Coulter counter.

Results

Breast Cancer Cells Exhibit Highly Varied ISG15 Expression, CPT Sensitivity, and TOP1 Down-Regulation

CPT sensitivity has been shown to vary greatly among various breast cancer cells *in vitro* (7, 8). No single variable could account for the observed variation in CPT sensitivity (8). In the present study, the hypothesis that the expression levels of ISG15 and its conjugates are correlated with CPT sensitivity of breast cancer cells was tested. As shown in Fig. 1, ZR-75-1 cells were much more (>50-fold) sensitive to CPT than BT474 cells based on cell counting (1-h acute exposure; Fig. 1A) and MTT (4 days continuous exposure; Fig. 1B). These results are consistent with previous published studies (7). As shown in Fig. 1C, breast cancer ZR-75-1 cells expressed high levels of ISG15 and its conjugates, whereas breast BT474 cells expressed very low levels of ISG15 and its conjugates. To test whether this apparent correlation between ISG15 and CPT sensitivity is generally true, other cells such as glioblastoma, colorectal cancer, and ATM+/ATM- cells were also included in the comparison. Indeed, there was a general correlation between ISG15 expression (Fig. 2A-C, *right*) and CPT sensitivity (Fig. 2D, IC_{50} determined by MTT assay) among these limited numbers of cell lines; cells expressing higher levels of ISG15 were more sensitive to CPT.

Previous studies have shown that the ISG15 pathway interferes with the ubiquitin/26S proteasome pathway (20). It is thus possible that elevated expression of the ISG15 pathway in these cells could lead to increased CPT sensitivity by interfering with proteasomal degradation of TOP1-DNA covalent complexes (TOP1 down-regulation). To test this hypothesis, CPT-induced degradation of TOP1 in these cells was measured. As shown in Figs. 1D and Fig. 2A to C (*left*), cells expressing high levels of ISG15 were much less efficient in CPT-induced TOP1 down-regulation. These results thus are consistent with the notion that the ISG15 pathway may affect CPT sensitivity through its interference with TOP1 down-regulation.

Elevated ISG15 Expression in Tumor Cells Confers Increased CPT Sensitivity

To show a causal relationship between ISG15 expression and CPT sensitivity, shRNA-mediated knockdown of ISG15 in breast cancer ZR-75-1 cells was conducted. Several clones of ISG15 shRNA transfectants and control shRNA transfectants were isolated (see Materials and Methods). One clone, ZR/ISG15-shRNA1, was extensively characterized. As shown in Fig. 3A, the expression level of ISG15 in ZR/ISG15-shRNA1 cells was significantly (>70%; as quantified by using the Image Station 2000R) reduced compared with that in ZR/control-shRNA1 cells. ZR/ISG15-shRNA1 cells were shown to be much more resistant to CPT (1-h acute exposure followed by cell survival measurement using clonogenic assay) than control-shRNA cells (Fig. 3B), suggesting that the ISG15 expression in tumor cells may be causally linked to CPT sensitivity.

The mechanism for increased CPT sensitivity in ISG15-overexpressing tumor cells was investigated. As suggested from results shown in Figs. 1 and 2, ISG15 overexpression is inversely correlated with TOP1 down-regulation. To test whether elevated ISG15 expression in tumor cells is causally linked to reduced TOP1 down-regulation, TOP1 down-regulation was measured in both ZR/ISG15-shRNA1 and ZR/control-shRNA1 cells. As shown in Fig. 3C, similar to ZR-75-1 cells (7), ZR/control-shRNA1 cells exhibited minimal TOP1 down-regulation. By contrast, ZR/ISG15-shRNA1 cells exhibited greatly increased rate of TOP1 down-regulation (~50% down-regulation in 2 h), suggesting that ISG15 negatively regulates TOP1 down-regulation. The level of TOP1 protein and the amount of TOP1 cleavable complexes formed in response to CPT treatment in the ZR/control-shRNA1 were not significantly different from those in ZR/ISG15-shRNA1 cells (data not shown). Hence, the observed differences in their CPT sensitivity and TOP1 down-regulation are not due to alteration in their

steady-state levels of TOP1 or the amount of TOP1 cleavable complexes in these cells. Together, these results suggest that reduced TOP1 down-regulation may contribute to increased CPT sensitivity in ISG15-overexpressing tumor cells.

Protein ISGylation in Tumor Cells Increases CPT Sensitivity

The above studies have suggested that the elevated ISG15 protein level in tumor cells could contribute to CPT sensitivity by interfering with TOP1 down-regulation. Previous studies have shown that, in addition to free ISG15 protein, the formation of ISG15-protein conjugates (ISGylation) also interferes with protein polyubiquitination (20). Therefore, the hypothesis that ISGylation is related to increased CPT sensitivity in ZR-75-1 cells was tested by knocking down the major E2, UbcH8, for ISG15. Several stable clones of UbcH8-shRNA transfected ZR-75-1 cells were isolated. Two clones, ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2, were extensively characterized.

As shown in Fig. 4A, the expression levels of UbcH8 in ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2 cells were reduced to 70% and 85%, respectively, compared with those in ZR/control-shRNA2 cells (another control shRNA clone isolated from control shRNA-transfected ZR-75-1 cells). The levels of ISG15-protein conjugates in ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2 cells were also significantly reduced compared with those in control-shRNA2 cells. These results confirmed the effectiveness of UbcH8 shRNA in knocking down UbcH8 in ZR-75-1 cells.

CPT sensitivity of ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2 clones was measured by clonogenic survival following 1-h acute CPT exposure. As shown in Fig. 4C, both ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2 cells were much more resistant to CPT than ZR/control-shRNA2, suggesting that the formation of ISG15-protein conjugates (ISGylation) affects CPT sensitivity.

TOP1 down-regulation in these clones was also determined. Similar to ZR-75-1 cells (7), ZR/control-shRNA2 cells exhibited minimal TOP1 down-regulation (Fig. 4D, *first panel*). By contrast, ZR/UbcH8-shRNA1 (Fig. 4D, *second panel*) and ZR/UbcH8-shRNA2 (Fig. 4D, *third panel*) cells exhibited significantly increased rate of TOP1 down-regulation (~50% degradation of TOP1 in 2 h). Again, the level of TOP1 protein and the amount of TOP1 cleavable complexes formed in response to CPT treatment in the ZR/control-shRNA2 were not significantly different than those in the two clones of ZR/UbCh8-shRNA (data not shown). Hence, the observed differences in the CPT sensitivity and TOP1 down-regulation are not due to the alteration in the steady-state levels of TOP1 or the amount of TOP1 cleavable complexes in these cells. Together, these results suggest that ISGylation in tumor cells interferes with TOP1 down-regulation and therefore increases CPT sensitivity.

ISG15 Expression Is Down-regulated in Tumor Cells Selected for CPT Resistance

Our results have shown that ISG15 and its conjugates are important determinants for intrinsic CPT sensitivity in various tumor cells. To test whether altered regulation of ISG15 might also contribute to acquired CPT resistance, the ISG15 protein levels were measured in three tumor cell lines selected for high levels of CPT resistance (see Fig. 5B). As shown in Fig. 5A, ISG15 expression in these CPT-resistant cell lines (the ovarian cancer cell line 2774/RC, the

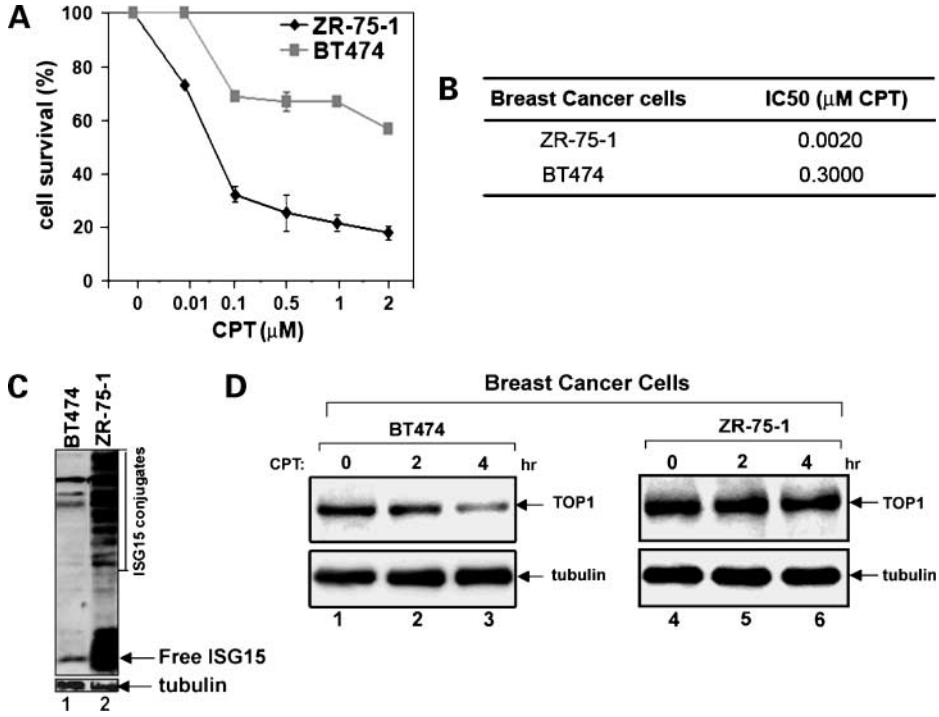

Figure 1. Breast cancer cells exhibit highly varied ISG15 expression, CPT sensitivity, and TOP1 down-regulation. **A,** CPT sensitivity. Breast cancer ZR-75-1 and BT474 cells were treated with CPT for 1 h followed by incubation in CPT-free medium for 4 d. Cell survival was determined by cell counting using a Coulter counter as described in Materials and Methods. **B,** CPT sensitivity. MTT assay was done at least twice in six replicate wells as described in Materials and Methods. **C,** ISG15 expression. ISG15 expression in breast cancer BT474 and ZR-75-1 cells was determined by immunoblotting using anti-ISG15 antibody as described in Materials and Methods. **D,** CPT-induced down-regulation of TOP1. Breast cancer BT474 and ZR-75-1 cells (10^6 per sample) were treated with either 1% DMSO (*lanes 1* and *5*) or CPT (25 μmol/L in 1% DMSO; *lanes 2-4* and *6-8*) for various times at 37°C. TOP1 down-regulation was then measured as described in Materials and Methods. The same membrane was stripped and reprobed with anti-tubulin antibody to verify equal protein loading (*bottom*).

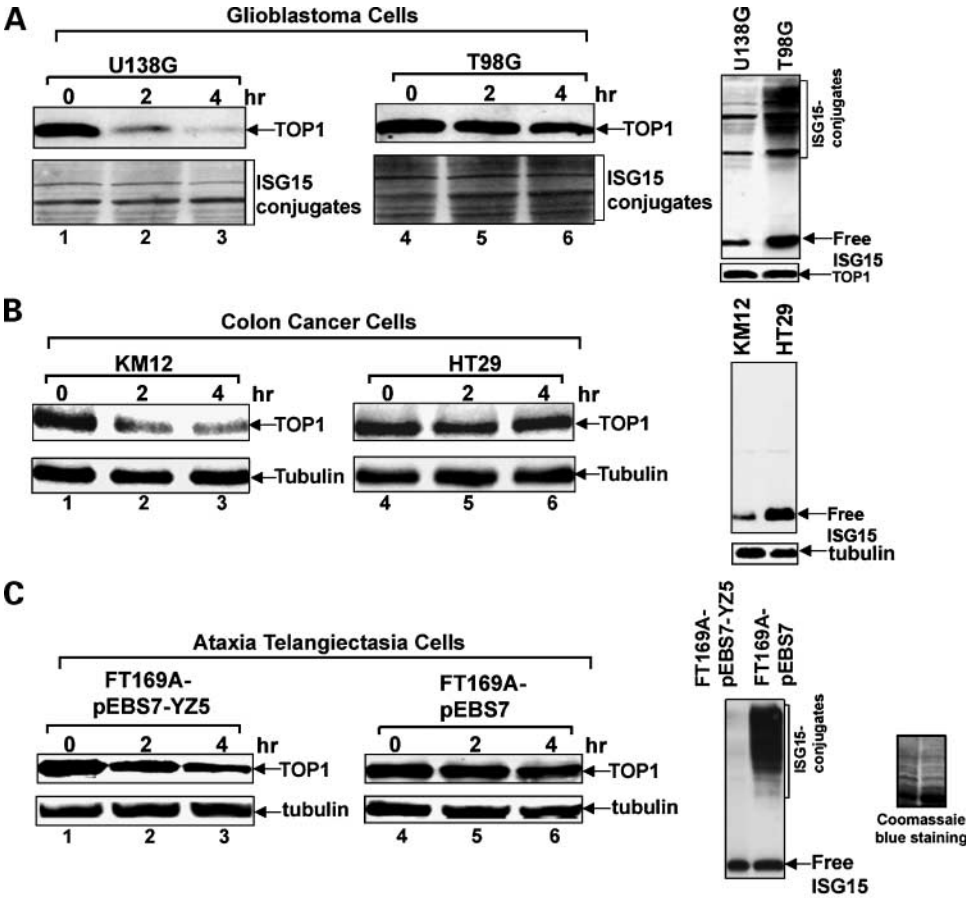

Figure 2. Elevated ISG15 expression in tumor cells is inversely correlated with TOP1 down-regulation. **A** to **C,** ISG15 expression and TOP1 down-regulation. Both ISG15 expression (*right*) and CPT-induced TOP1 down-regulation (*top left*) were determined in glioblastoma U138G and T98G cells (**A**), colorectal cancer HT29 and KM12 cells (**B**), and FT169A-pEBS7 (ATM-) and FT169A-pEBS7-YZ5 (ATM+) cells (**C**). Cells were treated with either 1% DMSO (*lanes 1* and *4*) or CPT (25 μmol/L in 1% DMSO; *lanes 2, 3, 5*, and *6*) for various times at 37°C. TOP1 down-regulation and ISG15 expression were measured as described in Materials and Methods. The same membranes shown in **A** to **C** (*top left*) and **A** and **B** (*top right*) were stripped and reprobed with anti-ISG15 (**A,** *bottom left*) or anti-tubulin (**B** and **C,** *bottom left*) and anti-TOP1 (**A,** *bottom right*) or anti-tubulin (**B,** *bottom right*) antibodies, respectively, to confirm equal protein loading. A duplicate gel was stained with Coomassie blue to assess protein loading in **C** (*right*). **D,** CPT sensitivity for cells expressing different levels of ISG15. MTT assay was done at least twice in six replicate wells as described in Materials and Methods.

D

Cell lines	IC50 (μM CPT)
Gliblastoma cells	
T98G	0.0150
U138G	0.9000
Colon cancer cells	
HT29	0.0075
KM12	0.0250
Ataxia telangiectasia cells	
FT169A-pEBS7	0.0060
FT169A-pEBS7-YZ5	0.0200

prostate cancer cell line DU145/RC, and the melanoma cancer cell line Bro/RC) was greatly (>5-fold) reduced compared with that in their respective parental cells (2774, DU145, and BRO cells). In addition to 2774, DU145, and BRO cells, three other CPT-resistant cell lines (RPMI/CPT-K5, U937/CR, and SB1B/RC) were also examined for their ISG15 expression. These three resistant lines showed no change in ISG15 expression (RPMI/CPT-K5), minimally reduced ISG15 expression (U937/CR), or no detectable ISG15.[5] The growth rates of these CPT-resistant cells were comparable with those of their respective parental cells (data not shown). Together, these results suggest that ISG15 expression may play an important role in acquired CPT resistance.

The resistant clones described above were selected for high levels of CPT resistance with stepwise increase in CPT concentrations. Many genetic changes are likely to have occurred in these resistant clones, which may complicate the interpretation of the role of ISG15 expression in CPT resistance. Indeed, DU145/RC and 2774/RC cells express mutant TOP1 proteins that are defective in the formation of TOP1 cleavage complexes in response to CPT (14). To avoid this problem, MCF7 breast cancer cells were selected for low levels of CPT resistance. The MCF7/RC (IC_{50},

[5] Unpublished results.

0.030 μmol/L), one of the low-level CPT-resistant clones of MCF7, was ~8-fold more resistant to CPT than MCF7 cells (IC_{50}, 0.0035 μmol/L). No change in the amount of TOP1-DNA covalent complexes was observed (Fig. 6A, *first panel*). However, ISG15 protein band at 15 kDa in MCF7/RC was significantly reduced compared with that in MCF7 cells when detected using either anti-ubiquitin (Fig. 6A, *third panel*, detected as a 15-kDa band labeled UCRP) or anti-ISG15 antibody (Fig. 6A, *fourth panel*, detected as ISG15). By contrast, a ubiquitin conjugate band (marked by an *asterisk*), which was detected by the anti-ubiquitin antibody (Fig. 6A, *third panel*), remained unchanged, suggesting that the reduced levels of UCRP (ISG15) is not due to reduced protein loading. Interestingly, TOP1 down-regulation, which was minimal in the parental MCF7 cells, was proficient in MCF7/RC cells (Fig. 6A, *second panel*).

Similar to MCF7 cells, breast cancer ZR-75-1 cells were also selected for low-level CPT resistance (selected with either 10 or 20 nmol/L CPT). Clones were isolated. Among 18 CPT-resistant clones, 7 of them showed reduced ISG15 expression. One of the CPT-resistant clones with reduced ISG15 expression (Fig. 6B, *right*) was further characterized for TOP1 down-regulation. As shown in Fig. 6B (*left*), CPT treatment was shown to induce TOP1 down-regulation only in CPT-resistant ZR-75-1 cells but not in parental ZR-75-1 cells. Together, these results suggest that ISG15 expression is an important determinant for acquired CPT resistance and provide additional support for the negative regulatory role of ISG15 in TOP1 down-regulation.

Discussion

It has been documented that CPT sensitivity varies greatly in different tumor cell lines and no single cellular variable has been shown to correlate with CPT sensitivity (6–8). It has also been shown that the expression of ISG15 and its conjugates is highly elevated in many tumors compared with their normal counterparts (20, 21). In the present study, the expression of ISG15 and its conjugates is shown to correlate with CPT sensitivity among several pairs of cancer cell lines (ZR-75-1 versus BT474 breast cancer cells, HT29 versus KM12 colorectal cancer cells, and T98G versus U138G glioblastoma cells).

The results from this limited correlation study are further supported by shRNA-mediated knockdown studies, confirming the causal relationship between the ISG15 conjugation pathway and CPT sensitivity. Two separate knockdown studies have been done in ZR-75-1 breast cancer cells, which are known to express a high level of ISG15 and be hypersensitive to CPT (20). First, shRNA-mediated knockdown of ISG15 in ZR-75-1 cells resulted in reduced CPT sensitivity. Second, shRNA-mediated knockdown of UbcH8 (the major E2 for ISG15) in ZR-75-1 cells also resulted in reduced CPT sensitivity. These results suggest that the formation of ISG15-protein conjugates (ISGylation) could be responsible for increased CPT sensitivity.

Previous studies have shown that shRNA-mediated knockdown of either ISG15 or UbcH8 results in increased protein polyubiquitination (20). It has been suggested that

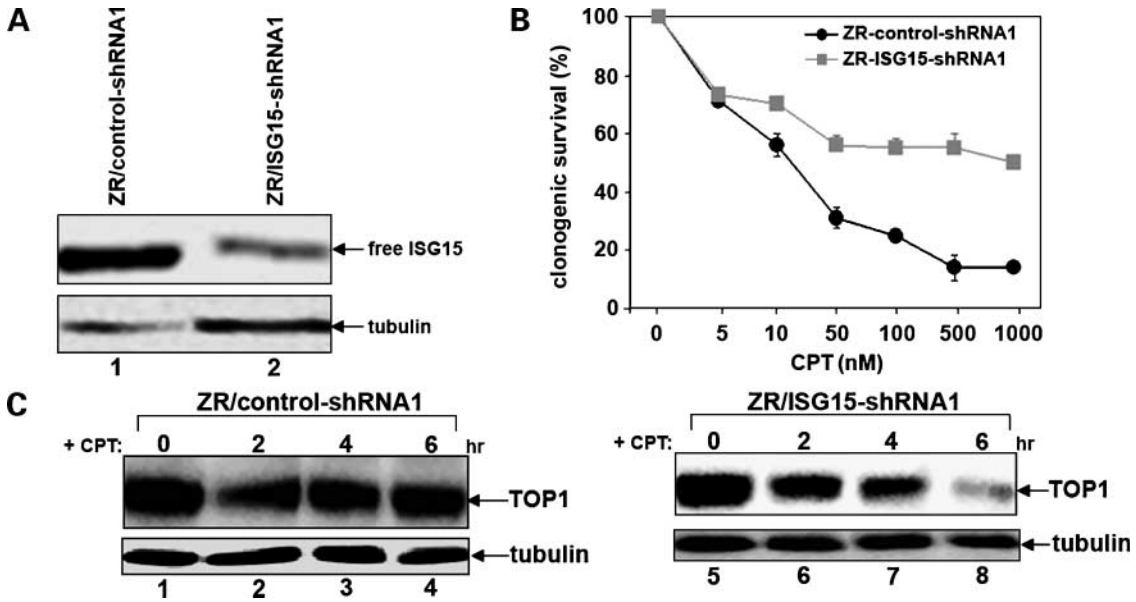

Figure 3. shRNA-mediated down-regulation of ISG15 in breast cancer ZR-75-1 cells confers CPT resistance and restores CPT-induced TOP1 down-regulation. **A,** reduced ISG15 expression in ZR-75-1 cells expressing ISG15 shRNA. Cell lysates of ZR-75-1 clones expressing either control shRNA (*lane 1*) or ISG15 shRNA (*lane 2*) were analyzed by 15% SDS-PAGE. Western blotting analysis was done using anti-ISG15 antisera (*top*). **B,** CPT sensitivity. Clonogenic survival assay was carried out as described in Materials and Methods. Colonogenic survival curves of a ZR-75-1 clone expressing control shRNA and ISG15 shRNA after CPT treatment. Mean ± SD from at least two experiments. **C,** CPT-induced TOP1 down-regulation. ZR-75-1 cells stably transfected with control shRNA (*lanes 1-4*) or ISG15 shRNA (*lanes 5-8*; 10^6 per sample) were treated with either DMSO (*lanes 1* and *5*) or CPT (25 μmol/L in 1% DMSO; *lanes 2-4* and *6-8*) for various times at 37°C. TOP1 down-regulation was measured as described in Materials and Methods.

protein ISGylation interferes with protein polyubiquitination, leading to reduced degradation of many proteins (20). Indeed, ISG15 expression has been shown to inhibit ubiquitination of Gag and Tsg101 proteins (proteins required for HIV-1 replication; ref. 37). Consistently, our present study has shown that shRNA-mediated knockdown of either ISG15 or UbcH8 results in increased proteasomal degradation of TOP1 in CPT-treated ZR-75-1 cells. Because CPT-induced proteasomal degradation of TOP1 (TOP1 down-regulation) has been suggested to be a repair mechanism for CPT-induced DNA lesion, it seems reasonable to speculate that elevated protein ISGylation in tumor cells may inhibit CPT-induced TOP1 down-regulation, leading to increased CPT sensitivity.

The importance of the ISG15 conjugation pathway in CPT sensitivity/resistance is further supported from studies of cell lines selected for acquired CPT resistance. Among six cell lines stepwise selected for high levels of CPT resistance, three (2774/RC ovarian cancer cells, DU145/RC prostate cancer cells, and Bro/RC melanoma cells) showed significant reduction of ISG15 expression compared with their respective wild-type cells. The other three showed either minimal change in ISG15 expression (RPMI/CPT-K5 and U937/CR) or undetectable ISG15 (SB1B/RC melanoma cells) compared with their respective wild-type cells.[5] The interpretation of the results could be complicated by the possibility that multiple genetic changes are likely to have occurred in these resistant clones. For example overexpression of drug efflux pump BCRP has been shown to be associated with CPT resistance (5). Altered TOP1 localization (5) or mutations in TOP1 has also been shown to lead to CPT resistance (5). To avoid these potential complications, we selected MCF7 and ZR-75-1 breast cancer cells for low-level CPT resistance. We observed that MCF7 and ZR-75-1 cells selected for low-level resistance also exhibit reduced ISG15 expression. Analysis of the low-level CPT-resistant clones of ZR-75-1 cells has shown that about one third of the clones exhibit reduced ISG15 expression. This is significant because low-level CPT resistance is more likely to mimic CPT resistance in patients. Our results thus support the possibility that the reduced expression of ISG15 could be one of the significant determinants of CPT resistance in the clinic.

Our results can be summarized schematically as shown in Fig. 6C. In this schematic, CPT induces TOP1-DNA

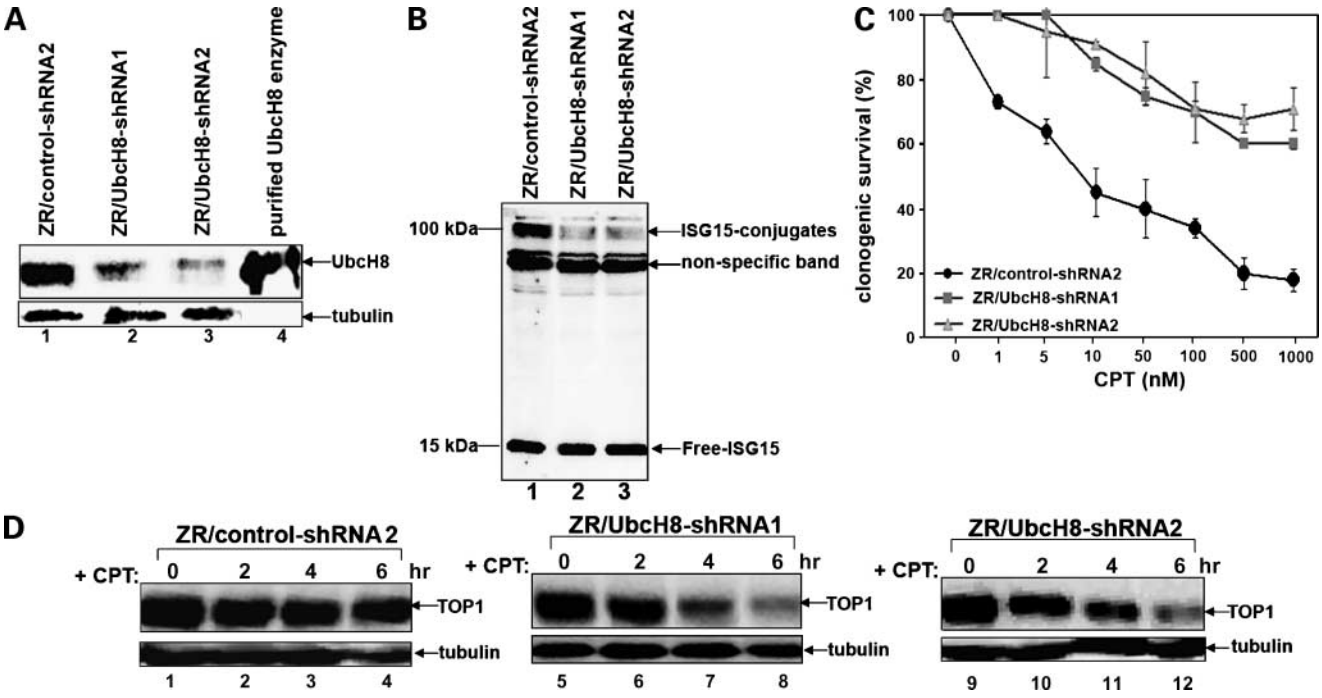

Figure 4. shRNA-mediated down-regulation of UbcH8 in breast cancer ZR-75-1 cells confers CPT resistance and restores CPT-induced TOP1 down-regulation. **A,** reduced UbcH8 protein level in ZR-75-1 cells expressing UbcH8 shRNA. Cell lysates prepared from ZR-75-1 clones expressing either control shRNA (*lane 1*, labeled ZR/control-shRNA2) or UbcH8 shRNA (*lanes 2* and *3*, from two independent clones, labeled ZR/UbcH8-shRNA1 and ZR/UbcH8-shRNA2, respectively) and the purified UbcH8 protein (*lane 4*) were analyzed by 15% SDS-PAGE. Western blotting analysis was done using anti-UbcH8 antibody (Abcam). **B,** UbcH8 shRNA decreases ISG15 conjugation in ZR-75-1 cells. Cell lysates prepared from ZR-75-1 clones expressing either control shRNA (*lane 1*) or UbcH8 shRNA (*lanes 2* and *3*) were analyzed by 15% SDS-PAGE. Western blotting analysis was done using anti-ISG15 antibody. The nonspecific bands are due to the cross-reactivity of the ISG15 antibody to bovine serum albumin. **C,** knocking down UbcH8 confers CPT resistance. ZR-75-1 clones expressing control shRNA and UbcH8 shRNA (two independent clones) were treated with CPT and cell survival was measured as described in Material and Methods. **D,** CPT-induced TOP1 down-regulation in ZR-75-1 cells expressing UbcH8 shRNA. Clonal cells of ZR-75-1 expressing control (*panel A, lane 1*) or UbcH8 shRNA (*panel A, lanes 2* and *3,* representing two independent clones; 10^6 per sample) were treated with either 1% DMSO (*lanes 1, 5*, and *9*) or CPT (25 μmol/L in 1% DMSO; *lanes 2-4, 6-8*, and *10-12*) for various times at 37°C. TOP1 down-regulation was measured as described in Materials and Methods.

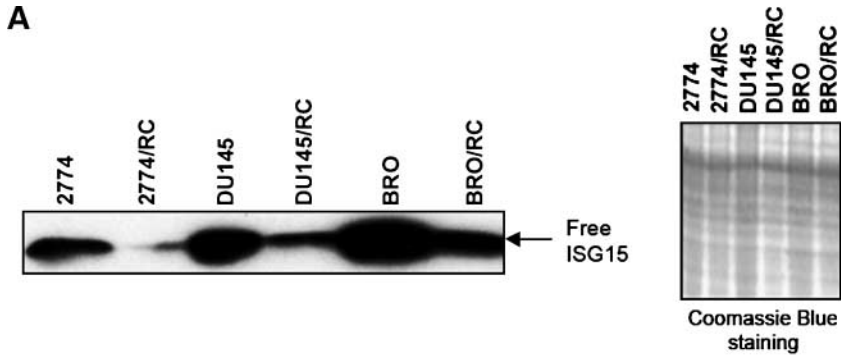

Figure 5. Reduced ISG15 expression in various tumor cells selected for high levels of CPT resistance. **A,** expression of ISG15 is reduced in tumor cells selected for CPT resistance. The human ovarian cancer 2774 and its CPT-resistant variant 2774/RC, the human prostate cancer DU145 and its CPT-resistant variant DU145/RC, and melanoma BRO and its CPT-resistant variant Bro/RC cells were analyzed by 15% SDS-PAGE followed by immunoblotting with anti-ISG15 antibody (*left*). A duplicate gel was stained with Coomassie blue to assess protein loading (*right*). **B,** determination of IC_{50} by MTT assay. Cells were treated with different concentrations of CPT for 4 days. MTT assay was then done as described in Materials and Methods.

B

Cell line	IC50 (μM)	
	CPT	Vinblastine
Ovarian cancer cells		
2774	0.004	0.0003
2774/RC	>10	0.0002
Prostate cancer cells		
DU145	0.004	0.0004
DU145/RC	6.0	0.0004
Melanoma cells		
BRO	0.007	0.0003
BRO/RC	0.5	0.0004

covalent complexes that are the key DNA lesion responsible for tumor cell killing. Ubiquitin/26S proteasome-mediated degradation of TOP1-DNA covalent complexes represents a repair mechanism for cell survival. During tumorigenesis, the ISG15 conjugation pathway is highly, but variably, elevated in tumors, resulting in reduced proteasomal degradation of TOP1-DNA covalent complexes and hence increased CPT sensitivity (Fig. 6C). The reason for elevated expression of the ISG15 pathway in tumors is currently being investigated.

Our results have shown a significant role of ISG15 in determining CPT sensitivity/resistance. These results

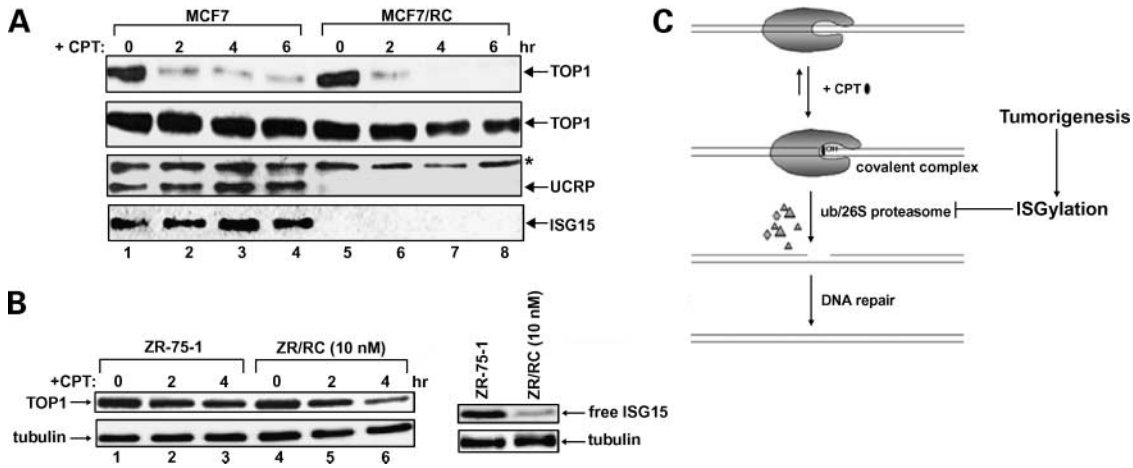

Figure 6. Reduced ISG15 expression in breast cancer cells selected for low levels of CPT resistance. **A,** MCF7 and MCF7/RC cells were treated with either 1% DMSO (*lanes 1* and *5*) or CPT (25 μmol/L in 1% DMSO; *lanes 2-4* and *6-8*) for 0, 2, 4, and 6 h. The amount of TOP1 cleavage complexes (*first panel*), the degree of TOP1 down-regulation (*second panel*), the free ISG15 levels detected as UCRP by using anti-ubiquitin antibody (*third panel*), and the free ISG15 levels detected by anti-ISG15 antibody (*fourth panel*) were determined by immunoblotting as described in Materials and Methods. **B,** CPT-induced TOP1 down-regulation in ZR-75-1 and ZR-75-1/RC (10 nmol/L clone 1) cells (*left*). ZR-75-1 cells and a stable CPT-resistant clone of ZR-75-1 cells (ZR/RC 10 nmol/L clone 1) were treated with either 1% DMSO (*lanes 1* and *4*) or CPT (25 μmol/L in 1% DMSO; *lanes 2, 3, 5*, and *6*) for 2 and 4 h. TOP1 down-regulation was measured as described in Materials and Methods. The expression levels of ISG15 in ZR-75-1 and ZR-75-1 10 nmol/L clone 1 (*right*). **C,** a proposed model for the role of protein ISGylation in CPT sensitivity/resistance. In this model, CPT is shown to trap the TOP1-DNA covalent complexes on chromosomal DNA. An ubiquitin/26S proteasome pathway is activated for the repair of these complexes by degrading TOP1. During tumorigenesis, the ISG15 conjugation pathway is elevated, which interferes with the ubiquitin-mediated degradation of TOP1, leading to increased CPT sensitivity.

could have significant implications in the clinic for cancer patients. The highly variable expression of ISG15 and its conjugates in tumors could be used as predictors for cancer cell sensitivity to CPT. This is particularly important because both ISG15 expression levels and CPT sensitivity are highly variable among different tumors (6–8). It is also interesting to note that, in addition to being present in tumors, ISG15 has been shown to be secreted by tumor cells (22) and detectable in blood (38). The blood ISG15 level may therefore be conveniently used for both diagnosis of cancer and prediction for CPT (and other TOP1-directed drugs) treatment response in targeted chemotherapy.

Modulation of the ISG15 pathway also could have implications in drug combinations in the clinic. Because type I IFN are known to induce the ISG15 pathway (22, 23, 26), a combined use of type I IFN and CPT (or other TOP1-targeting drugs) is expected to increase CPT sensitivity in tumors expressing low levels of ISG15. Indeed, IFN have been shown to exhibit synergistic anticancer activity with CPT-11 against human colon cancer xenografts in nude mice (39, 40). It is also interesting to note that all-*trans* retinoic acid is known to induce ISG15 expression possibly through type I IFN (41). Consequently, all-*trans* retinoic acid, like type I IFN, could be used in combination with CPT (or other TOP1-targeting drugs) to sensitize tumors with low levels of ISG15 expression. Clearly, further studies on the molecular basis for the connection between ISG15 and CPT sensitivity/resistance may have important clinical implications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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